Development of rheumatoid arthritis is not associated with two polymorphisms in the Crohn’s disease gene CARD15

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Introduction. It has been proposed that genetic susceptibility loci for rheumatoid arthritis (RA) may be shared with other autoimmune/inflammatory diseases. Recently, common variation in the CARD15 (NOD2) gene on chromosome 16q12 has been associated with Crohn’s disease (CD) in several independent populations. CARD15 is an excellent functional and positional candidate gene for RA.

Methods. Genomic DNA was obtained from 392 RA cases and 471 ethnically matched healthy controls. All samples were genotyped for two polymorphisms in CARD15, 1007fs and R702W, using 5' nuclease reporter assays. Allele frequencies were compared between cases and controls using the χ² test. Estimated haplotype frequencies across the two mutations were determined using the EH program.

Results. The allele frequency of the 1007fs variant in RA cases was 1.8% compared with 1.6% in normal controls (not significant). The frequency of the R702W variant was 4.0% in both cases and controls. Haplotypes carrying either of the two mutations accounted for 5.6% of possible haplotypes. A haplotype carrying both mutations was rare, with estimated frequency <0.01%. This study provided high power to detect an association of similar magnitude to that in Crohn's disease. These data therefore exclude the possibility that the contribution of these mutations to RA is comparable to that seen in CD.

Conclusion. Within defined statistical parameters, we excluded a role for the CARD15 1007fs and R702W variants in RA susceptibility. These data do not preclude a role for other polymorphisms in the CARD15 gene in RA susceptibility. Results from other autoimmune and inflammatory diseases will reveal whether the CARD15 gene is in fact a common autoimmune susceptibility locus.

Rheumatoid arthritis (RA) is a typical complex multifactorial disorder in which unknown environmental and incompletely characterized genetic factors interact to cause disease [1]. Heritability estimates suggest that 60% of the population’s liability to develop RA may be genetically determined [2]. The HLA region is estimated to account for 30–40% of this, indicating that the majority of the genetic component of disease susceptibility lies outside the region [3]. A number of different strategies are being used to localize this component, which is likely to consist of several genes, each with a small disease effect. Linkage studies have been carried out in multi-case families (mostly affected sibling pairs) and have taken the form of genome-wide screens or have followed a candidate gene approach [4–6]. The role of individual candidate genes has also been investigated in association studies (using single-case and multicase families or case-control pairs), which often have greater power to detect weak gene effects [7]. To localize variation accounting for disease, positive linkage results are usually followed by testing for association between disease and genetic polymorphism(s) in candidate genes.
mapping to the identified region. This approach has enabled the analysis of a specific genetic variant in RA; the interleukin 1 (IL-1) gene cluster on chromosome 2q13, the corticotropin releasing hormone (CRH) genomic region on 8q12.3 and the tumour necrosis factor receptor II (TNFR II) locus on 1p36 [8–11].

An additional method of identifying candidate genes is to draw parallels with other autoimmune or inflammatory diseases thought likely to have a similar underlying pathogenesis and complex genetic basis. A meta-analysis of 23 published genome-wide scans revealed clustering of non-HLA susceptibility genes in human autoimmune disease [12]. This supports the hypothesis that clinically distinct autoimmune diseases may be controlled by a common set of susceptibility genes. Myerscough et al. [13] tested for linkage and association between five insulin-dependent diabetes mellitus (IDDM) susceptibility loci and RA and found evidence of association between markers at IDDM5 and IDDM8 with disease. Crohn's disease (CD) is another example of a complex multifactorial disease, with epidemiological and linkage data supporting a significant genetic component in disease aetiology. Three groups have independently reported association of disease with polymorphisms in the NOD2 gene (now known as CARD15), located at 16q12 [14–16]. Linkage of RA with a broad region of the long arm of chromosome 16 (16q22–16q24) has recently been reported by the North American Rheumatoid Arthritis Consortium and was also found by the European Consortium on Rheumatoid Arthritis Families at 16q24 [4, 6]. It is acknowledged that the map resolution of the non-parametric linkage approach is limited. The physical distance between the CARD15 locus and these linked markers is between 31 and 38 megabases. Given the standard errors of genetic location estimates of tens of centimorgans for weak candidate susceptibility loci [17], CARD15 is a reasonable candidate for genetic association studies for RA. This is strengthened by the evidence of its role in activating nuclear factor NF-κB in response to bacterial lipopolysaccharide in mononuclear cells. This renders it an excellent functional candidate for RA as well. The insertion polymorphism associated with CD [14–16] results in truncation of the leucine-rich region of the gene product. This mutant CARD15 is significantly less active than the wild-type in conferring responsiveness to bacterial lipopolysaccharide [16]. For the reasons outlined, we tested for genetic association between RA and two CARD15 polymorphisms.

Methods

Patient and control characteristics
Genomic DNA was obtained from 289 sporadic cases of RA recruited from the Oxfordshire and Southwest UK regions and from an additional 103 RA cases identified in the course of recruiting single-case RA families from the same regions. All patients satisfied the American College of Rheumatology 1987 criteria for RA. Regional ethics committee approval was obtained, and written, informed consent was given by all participants. Genomic DNA was also obtained from 471 healthy ethnically matched controls; samples from 186 subjects were from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, England), 192 participants were recruited through the Tissue Typing laboratory at Guy's Hospital and 93 were recruited from residents in the Newcastle area as part of a regional genetic study.

Genotyping
All cases and controls were genotyped for two single-nucleotide polymorphisms (SNPs) in the CARD15 gene: (i) a frameshift C-insertion mutation, 3020insC (1007fs), in exon 11, which results in a truncated gene product, and (ii) a missense mutation, 2104C→T (R702W), in exon 4. These sequence alterations were previously reported as SNP13 and SNP8 (reference SNP ID rs2066847 and rs2066844 respectively) [15]. Both polymorphisms were genotyped using the Taqman 5′ nuclease biallelic discrimination system [18] in conjunction with an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The 1007fs polymorphism was typed with primers 5′-GTCCAATAACTGCATCACCATACTTAG-3′ (forward), 5′-CCCTCCTGAGGAGCTTGCCT-3′ (reverse) and probes 5′-CCCTCCTGAGGAGCTTGCCT-3′ (wild-type) and 5′-CTCTCTGACCCTCCCCCTCTAGA-3′ (mutant). For R702W the primers were 5′-GCTGGCTGAGTGCAGACATC-3′ (forward), 5′-AGTGGAGATGCGTGTGGAGG-3′ (reverse) and probes 5′-CCTGGTCTCCGGGCGCCAGGC-3′ (wild-type) and 5′-CCTGGTCTCCGGGGCGCCAGGC-3′ (mutant). Genotypes were assigned manually.

Statistical analysis
Tests for Hardy–Weinberg equilibrium (HWE) and case–control association analyses were performed using the χ2 test. Linkage disequilibrium was assessed using the coefficient D′ as a measure of association [19]. Haplotype frequencies were estimated from 347 cases and 447 controls using the EH program [20]. The power of this study was described in terms of disease odds ratios, which were estimated from expected genotype frequencies assuming HWE.

Results
Genotypes for each of the SNPs were in HWE in both cases and controls. No significant differences in the distribution of genotypes were observed between the control groups from ECACC, Guy's Hospital or Newcastle; these data were therefore pooled for subsequent analysis. The frequency of the 1007fs variant in RA cases was 1.8% compared with 1.6% in normal controls (not significant). Similarly there was no evidence for association of R702W with RA; the frequency of the variant in both cases and controls was 4.0%.

No evidence of linkage disequilibrium between 1007fs and R702W was observed (coefficient D = −0.02). Estimated haplotype frequencies across the two mutations were determined in 347 RA cases and 447 healthy controls, as shown in Table 1. Haplotypes carrying either of the two mutations account for 5.3% of possible haplotypes. A haplotype carrying both mutations is rare, with estimated frequency <0.01%; this is consistent with the findings in CD [21]. Genotypes were therefore described in terms of the three possible
CARD15 haplotypes (wild-type, 1007fs, R702W). For each of the individual polymorphisms there was no significant difference in the frequency of these composite CARD15 haplotypes between cases and controls. Genotype and mutation frequencies for cases and controls are displayed in Table 2.

For a mutation occurring with frequency 1.6% in the general population (1007fs), a case–control study with sample sizes comparable to ours provides 80% power to detect a difference in mutation frequency if the true frequency in the RA case population was 4%. This corresponds to a disease odds ratio of 2.58 associated with carrying at least one copy of the mutation. The equivalent odds ratio, assuming a control frequency of 4% for R702W, is 2.04. If, as in CD, these mutations contribute independently to disease risk and the odds ratio associated with this composite CARD15 haplotype is 1.91, this study provides 80% power to detect an effect of CARD15 in RA.

### Discussion

In this study of 392 RA cases and 471 normal controls, two polymorphisms in the coding region of the CARD15 gene that have been reported as being associated with CD were genotyped. We show an absence of association of these polymorphisms with RA. Positive findings in case–control studies are often vulnerable to the criticism of possible background genetic heterogeneity between the two populations, leading to a false-positive result [22, 23]. Such results, particularly when derived from small studies, are generally viewed with some scepticism, at least until independently replicated. Although this is unlikely to be a problem in a negative study of adequate size where the genotypic data show HWE, it is theoretically possible that this result may be a false negative and replication of this study would be useful in other populations.

A more serious criticism of negative studies is that they lack the power to show an association if it is present, usually because of inadequate sample sizes. To aid interpretation of studies it is important that the power of genetic association studies is stated explicitly. The power calculations presented here demonstrate that this study should have been able to detect an effect of these polymorphisms on disease risk if in combination they confer a 1.91-fold increase in the disease risk. The odds ratios for each of the polymorphisms individually were 2.04 (R702W) and 2.58 (1007fs). In CD the odds ratio for an individual heterozygous at R702W having disease was 2.66 and the corresponding value at 1007fs was 2.59 [21]. Therefore we are confident that we have excluded an effect for these two polymorphisms of similar magnitude to that seen in CD. To date, one other mutation in the CARD15 gene has been associated with CD, which contributes a magnitude of disease risk similar to that contributed by 1007fs [15, 21]. Other rare variants have also been identified in CD patients [24]. This study does not preclude an effect for other variants in the CARD15 gene but, given their low frequency in white Europeans, our power to detect such an effect would be very limited. It also does not exclude a weaker association of these polymorphisms with RA. To eliminate these possibilities and convincingly remove CARD15 from the list of possible candidate genes in RA, further larger studies are needed.

It has been suggested previously that distinct inflammatory and autoimmune diseases may be influenced by shared genetic loci [12, 25]. Several other complex diseases have been linked to or associated with the same region of chromosome 16. These include systemic lupus erythematosus [26–28], psoriasis [29], IDDM [30], ankylosing spondylitis [31] and Blau syndrome [32]. To date, CD is the only disease with a replicated association in this region. A recent report has shown no association between psoriasis and the 1007fs mutation [33] and similarly ulcerative colitis does not appear to be associated with this variant [21].

RA is characterized by chronic inflammation in which a crucial role for TNF-α has been demonstrated at both laboratory and clinical levels [34]. TNF-α is similarly important in CD. In the light of the known function of CARD15 in the activation of NF-κB in response to bacterial lipopolysaccharide, we reasoned that, on the basis of function and genetic location evidence, it is a plausible candidate for RA. The evidence we present suggests that, within a defined statistical framework, the two polymorphisms tested have no role in determining the risk of development of RA. Results from other
autoimmune and inflammatory diseases will reveal whether the \textit{CARD15} gene is in fact a common autoimmune susceptibility locus. Meanwhile, the peak of linkage at chromosome 16q22-24 in RA remains unexplained and clarification will require further work.

References